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GDF-15 contributes to proliferation and immune escape of malignant gliomas

Roth, P ; Junker, M ; Tritschler, I ; Mittelbronn, M ; Dombrowski, Y ; Breit, S N ; Tabatabai, G ; Wick, W ; Weller, M ; Wischhusen, J

Abstract: PURPOSE: Growth and differentiation factor (GDF)-15 is a member of the transforming growth factor (TGF)-beta family. GDF-15 is necessary for the maintenance of pregnancy but has also been linked to other physiological and pathological conditions. EXPERIMENTAL DESIGN: The expression of GDF-15 in glioma cell lines was assessed by qRT-PCR and immunoblot. GDF-15 levels in situ and in peripheral blood of glioma patients were examined by immunohistochemistry and ELISA, respectively. Effects of shRNA-mediated GDF-15 inhibition on proliferation and immunogenicity of SMA-560 glioma cells were investigated by [methyl-3H]thymidine incorporation and immune-mediated target cell lysis. The impact of GDF-15 on glioma growth in vivo was assessed in syngeneic mice.RESULTS: GDF-15 is expressed by gliomas of different WHO grades as assessed by immunohistochemistry. The high expression of GDF-15 in tumor tissue translates into elevated GDF-15 serum levels in glioblastoma patients compared to healthy controls. GDF-15 mRNA and protein are also detectable in human and mouse glioma cells in vitro. Silencing of GDF-15 by RNA interference reduces the proliferation of malignant glioma cells. Immunologically, the depletion of glioma-derived GDF-15 enhances the susceptibility of mouse glioma cells towards syngeneic NK cells and splenocytes. This results into a reduced in vivo tumorigenicity and increased T cell infiltration of GDF-15-deficient glioma cells in syngeneic mice. CONCLUSIONS: While previous studies focussing on ectopic overexpression of GDF-15 have proposed unclear or anti-tumorigenic effects of GDF-15 in glioma cells, we here show that GDF-15 at endogenous levels contributes to proliferation and immune escape of malignant gliomas in an immunocompetent host.

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GDF-15 contributes to proliferation and immune escape of malignant gliomas

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Running title: Tumor-promoting functions of GDF-15 in malignant gliomas

Key words: glioma, immune escape, proliferation, GDF-15

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TRANSLATIONAL RELEVANCE

The prognosis of patients afflicted by malignant glioma remains poor despite multimodal therapy. Therefore, there is an urgent need to dissect the cell-biological mechanisms that contribute to the malignant phenotype. Here, we demonstrate that gliomas express *growth and differentation factor* (GDF)-15 *in vitro* and *in vivo*. We provide evidence that GDF-15 contributes to proliferation and immune escape of glioma cells. Most importantly, we show that silencing of glioma-derived GDF-15 leads to prolonged survival in syngeneic mice. Consequently, we propose GDF-15 as a novel target for future therapeutic approaches against malignant gliomas.

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ABSTRACT

Purpose: Growth and differentiation factor (GDF)-15 is a member of the transforming growth factor (TGF)-β family. GDF-15 is necessary for the maintenance of pregnancy but has also been linked to other physiological and pathological conditions.

Experimental Design: The expression of GDF-15 in glioma cell lines was assessed by qRT-PCR and immunoblot. GDF-15 levels *in situ* and in peripheral blood of glioma patients were examined by immunohistochemistry and ELISA, respectively. Effects of shRNA-mediated GDF-15 inhibition on proliferation and immunogenicity of SMA-560 glioma cells were investigated by [methyl-^{3}H]thymidine incorporation and immune-mediated target cell lysis. The impact of GDF-15 on glioma growth *in vivo* was assessed in syngeneic mice.

Results: GDF-15 is expressed by gliomas of different WHO grades as assessed by immunohistochemistry. The high expression of GDF-15 in tumor tissue translates into elevated GDF-15 serum levels in glioblastoma patients compared to healthy controls. GDF-15 mRNA and protein are also detectable in human and mouse glioma cells *in vitro*. Silencing of GDF-15 by RNA interference reduces the proliferation of malignant glioma cells. Immunologically, the depletion of glioma-derived GDF-15 enhances the susceptibility of mouse glioma cells towards syngeneic NK cells and splenocytes. This results into a reduced *in vivo* tumorigenicity and increased T cell infiltration of GDF-15-deficient glioma cells in syngeneic mice.

Conclusions: While previous studies focussing on ectopic overexpression of GDF-15 have proposed unclear or anti-tumorigenic effects of GDF-15 in glioma cells, we here show that

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GDF-15 at endogenous levels contributes to proliferation and immune escape of malignant gliomas in an immunocompetent host.

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GDF-15, growth and differentiation factor-15; HRP, horseradish peroxidase; MACS, magnetic-activated cell sorting ; TGF-β, transforming growth factor-β.

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INTRODUCTION

Human glioblastomas are characterized by a very poor prognosis. Despite multimodal therapy, median survival is limited to less than 5 months in population-based studies (1). The dismal prognosis results from the infiltrative growth of chemoresistant glioma cells in the brain parenchyma. Thus, there is an urgent need for new therapeutic strategies. Harnessing the exquisite specificity and potent cytotoxicity of immune effector cells for tumor therapy should be a safe and potentially powerful strategy against cancer (2). A molecular basis for this approach is provided by our finding that glioblastoma cells express ligands which activate natural killer (NK) cells and provide co-stimulation for T cells (3). However, we and others have also identified a number of negative immune regulatory, glioma-derived factors that seem to dominate tumor-host interactions *in vivo*. These mediators of immune tolerance include the non-classical MHC molecules HLA-G and HLA-E (4-5), RTF, LLT-1 (6-7), IL-10 (8) and, most notably, TGF-β (9-10). Considering the overwhelming evidence that TGF-β substantially contributes to the deficits in cellular immunoreactivity displayed by human glioma patients, it is surprising that the potential immune-modulatory role of non-classical TGF-β superfamily members has so far hardly been investigated.

In this context, we have now analyzed the expression and potential function of *growth and differentiation factor* (GDF)-15 (11) in human and murine glioma cells. GDF-15 (alternative names: macrophage inhibitory cytokine 1 (MIC-1) (12), placental TGF-β (PTGF-β) (13), placental bone morphogenetic protein (PLAB) (14), prostate-derived factor (PDF) (15), non-steroidal anti-inflammatory drug-activated gene (NAG-1) (16), PL74 (17)), is a divergent member of the TGF-β superfamily which displays similarity with both bone morphogenetic proteins (31-35% homology) and classical TGF-β isoforms (~25% homology). It is

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synthesized as pro-protein (279 amino acids) which is secreted and proteolytically processed to yield the mature homo-dimeric cytokine (2x112 amino acids). Intriguingly, the cleaved pro-domain can bind to the extracellular matrix (18). Thus, both the pro-domain and the mature protein could exert biological functions.

Under physiological conditions, low to moderate levels of GDF-15 are expressed in most healthy tissues including brain (11, 19), liver, breast, colon (20) and bone marrow. However, much higher levels of GDF-15 are found in placenta (21) where it may help to prevent miscarriage (22). Additionally, GDF-15 overexpression has been described in numerous malignancies including breast, colorectal, pancreatic and prostate cancer (23-24). In melanoma, higher GDF-15 expression is seen in metastasis tissue than in primary tumor (25). Likewise, glioma patients with high CSF GDF-15 levels show shorter survival (26).

Functionally, GDF-15 may exert very heterogeneous functions in tumours. It protects prostate cancer cells against the cytotoxic effects of docetaxel and mitoxantrone (27). However, it can also act as a downstream mediator of apoptosis (28). A tumor suppressor function of GDF-15 became evident when C57BL/6J-Apc$^{-/+}$ mice were crossbred with mice that expressed a GDF-15 transgene. In this spontaneous model for intestinal neoplasia, GDF-15 overexpression reduced the formation of adenomas and polyps by about 60% (29). Likewise, in a nude mouse glioma model, GDF-15-transfected LN-Z308 cells lost their tumorigenicity (30). In contrast, GDF-15-overexpressing DU145 prostate cancer cells grew normally in nude mice. Interestingly, the high levels of GDF-15 were linked to tumor-related cachexia in these animals which correlates with clinical data from prostate cancer patients (31). Thus, the effect of GDF-15 on tumor growth seems to depend on the tumor stage and type. One major problem with respect to the cited studies is that they all investigate effects caused by artificial overexpression of GDF-15 which may not be observed at endogenous GDF-15 levels. Moreover, the potential impact of GDF-15 on anti-tumor immunity has not

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been addressed so far. Thus, we set out to investigate whether endogenous GDF-15 levels affect glioma growth *in vitro* and *in vivo* in the presence of a fully functional murine immune system.

MATERIALS AND METHODS

Cells and reagents

All human glioma cell lines were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) and have been characterized previously (32-33). Primary glioblastoma cells were established from freshly resected tumor tissue, cultured in monolayers and used between passages 4 and 9. SMA-560 glioma cells were obtained from D. D. Bigner (Duke University Medical Center, Durham, NC, USA). The cells were maintained in DMEM containing 10% FCS (Biochrom KG, Berlin, Germany) and penicillin (100 IU/ml)/streptomycin (100 µg/ml) (Gibco, Karlsruhe, Germany). The pSUPERpuro constructs targeting nucleotides 722 – 740 of murine GDF-15 NM_011819.1 were cloned as previously described (33). For the generation of stable shGDF-15 transfectants, pSUPERpuro control or shGDF-15 plasmids were introduced using FuGene6 transfection reagent (Roche, Mannheim, Germany). The cells were selected in medium containing 2 µg/ml puromycin (Sigma, Deisenhofen, Germany). Anti-GDF-15 antibody was prepared as described (12, 34).

Quantitative RT-PCR

Total RNA was prepared using the RNeasy system (Quiagen, Hilden, Germany) and transcribed according to standard protocols. For real-time PCR, cDNA amplification was monitored using SYBRGreen chemistry on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The conditions for these PCR reactions were: 40 cycles, 95°C/15 s, 60°C/1 min, using the following specific primers: 18S up: 5´-

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CGGCTACCACATCCAAGGAA-3′ (nucleotides 450-469), 18S down: 5′-GCTGGAATTACCGCGGCT-3′ (nucleotides 636-619); GDF-15 up: 5′-CCCTGCAGTCCGGATACTC-3′ (nucleotides 274-292), GDF-15 down: 5′-GAACAGAGCCCGGTGAAG-3′ (nucleotides 400-383). Data analysis was done using the $\Delta\Delta C_T$ method for relative quantification. Threshold cycles (C_T) for 18S rRNA (reference) and GDF-15 (sample) were determined in duplicates. We chose normal brain cDNA as calibrator tissue (100%) and determined the relative change (rI) in copy numbers according to the formula $rI = 2^{-[(C_T\text{GDF-15 normal brain} - C_T\text{18S normal brain}) - (C_T\text{GDF-15 glioma} - C_T\text{18S glioma})]}$.

Immunoblot

Supernatants were obtained from cells grown under serum-free conditions for 48 h. Soluble proteins > 3 kDa were concentrated using Vivaspin 20 columns (Vivascience, Hannover, Germany) before 20 µg/lane were separated on 15% acrylamide gels (Biorad, Munich, Germany). After transfer to nitrocellulose (Biorad), the blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20 and incubated overnight at 4°C with sheep anti-human MIC-1/GDF-15 antibody 233B3 (1:4,000) (12) which also recognizes the murine homologue. Visualization of protein bands was accomplished using HRP-coupled secondary antibodies (Sigma) and ECL (Amersham, Braunschweig, Germany). After scanning, relative signal intensities were quantified with Photoshop CS (Adobe, San José, CA, USA).

Immunohistochemistry

Gliomas of different WHO grade of malignancy were examined. Normal human brain white matter was used as control tissue, human placenta served as a positive control. GDF-15 immunohistochemistry was performed on formalin-fixed and paraffin-embedded samples.

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The polyclonal rabbit anti-human GDF-15 antibody was validated by the Swedish Atlas consortium and obtained via Sigma (#HPA011191).

Specimens were deparaffinized first in xylol followed by decreasing concentrations of ethanol before antigens were retrieved by microwave boiling in 10 mM citrate buffer (pH6) for 10 min. Endogenous peroxidases were blocked with 3% H_2O_2 in methanol for 10 min. To prevent unspecific binding, the sections were blocked for 15 min with beriglobin. A 1:50 dilution of anti-GDF-15 antibody in "antibody diluent" (DAKO, Hamburg, Germany) was applied over night at 4°C. The sections were then incubated with the biotinylated secondary goat anti-rabbit antibody for 15 min followed by a horseradish peroxidase-conjugated streptavidin incubation (both "ready to use" from DAKO) for another 15 min. Specificity was controlled by omission of primary antibody.

For visualization, the sections were incubated with diaminobenzidine (DAB) (DCS ChromoLine, Hamburg, Germany) in DAB substrate buffer (1:25 dilution) for 5 min, before they were washed and counterstained with haematoxylin, followed by a graded ethanol series. Finally, mounting was performed in Vitro-Clud® (R. Langenbrinck, Teningen, Germany).

ELISA

Sera of patients without prior exposure to chemotherapy or steroids and healthy control donors were obtained after informed consent. All diagnoses were confirmed by histology. For ELISA, MaxiSorp plates (Nunc, Wiesbaden, Germany) were coated overnight with 2 µg/ml of capture antibody (R&D Systems, #841832, Minneapolis, MN, USA) before the plates were washed and blocked. The pure or diluted samples were applied for 2 h at room temperature and bound GDF-15 was detected with biotinylated anti-GDF-15 detection antibody (R&D Systems, #841833) followed by streptavidin-HRP (R&D Systems) and

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3,3'5,5'-Tetramethylbenzidin (DAKO). The colour reaction was stopped by addition of 1M H_2SO_4 and absorbance was recorded in an ELISA reader (Tecan, Männedorf, Switzerland) at 450 nm. Using recombinant human GDF-15 as standard (R&D Systems), the assay was linear for GDF-15 concentrations between 0.1 ng/ml and 4 ng/ml.

For all other cytokine measurements, freshly isolated splenocytes (5×10^6) from VM/Dk mice were stimulated with SMA-560 control or shGDF-15 cells (5×10^5) for 3 days in 6 cm dishes. Supernatants were harvested and IL-2, IL-10 and IFN-γ concentrations were determined by ELISA according to the manufacturer's protocol (eBioscience, San Diego, CA, USA).

Glioma cell proliferation

5×10^3 SMA-560 control or shGDF-15 cells were plated in 96-well flat-bottomed plates and cultured in serum-free medium. Cultures were pulsed with [methyl-^{3}H]thymidine (1 μCi; Amersham) on day 2 and collected 16 h later using a cell harvester (Tomtec, Hamden, CT). Incorporated radioactivity was bound to a glass fibre filtermat (Wallac, Turku, Finland). The filtermat was wetted with Ultima Gold Scintillation Cocktail (Packard, Dreieich, Germany) and radioactivity was determined in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter.

Preparation of murine splenocytes and NK cells

Splenocytes were prepared from VM/Dk mice. Murine NK cells were positively selected using DX5 mAb-coupled magnetic beads with the corresponding column system (Miltenyi Biotech, Bergisch Gladbach, Germany). Polyclonal mouse NK cells were cultured with mouse IL-2 (5000 U/ml; PeproTech, Hamburg, Germany) for at least 10 days before being used as effector cells in cytotoxicity assays.

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Lysis assays

NK cell cytotoxicity was assessed in 4 h ^{51}Cr release assays with 10^4 $^{51}[CrO_4]^{2-}$-labeled targets per well and various effector:target (E:T) ratios in 100 µl of medium. Spontaneous ^{51}Cr release was determined by incubating the target cells with medium alone. To obtain the maximum ^{51}Cr release, NP-40 (2%) was added. After coincubation for 4 h, 50 µl of the supernatant were transferred to a Luma-Plate 96 (Packard, Dreieich, Germany), dried and measured. The percentage of ^{51}Cr release was calculated as follows: 100 x ([experimental release – spontaneous release]/[maximum release – spontaneous release]). The lytic activity of NK cell-depleted splenocytes was examined after 5 days of coculture with glioma cells. Irradiated glioma cells (5 x 10^5) were seeded into 6 cm dishes. Splenocytes (5 x 10^6) were added in 3 ml RPMI 1640 containing 10% FCS. Primed alloreactive splenocytes were removed at day 5 and used at different E:T ratios in a ^{51}Cr release assay as described above.

Mice and animal experiments

VM/Dk mice are bred in our own laboratory. Mice of 6–12 weeks of age were used in all experiments. The experiments were performed according to NIH guidelines, Guide for the Care and Use of Laboratory Animals. Groups of 6 mice were injected subcutaneously in the right flank with 10^6 transfected SMA-560 tumor cells in 0.1 ml PBS as indicated. Mice were examined regularly over 30 days for tumor growth using a metric caliper. Before all intracranial procedures mice were anesthetized by an intraperitoneal injection of 7% chloral hydrate. For intracranial implantation the mice were placed in a stereotactic fixation device (Stoelting, Wood Dale, IL, USA) and a burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. SMA-560 glioma cells ($5x10^3$) were injected in a volume of 2 µl PBS

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into the right striatum. The mice were observed daily and sacrificed when developing neurological symptoms. For immunohistochemical stainings, brain cryosections were prepared from mice sacrificed on day 15 after glioma cell inoculation. The following antibodies were used: anti-CD3 (BD Bioscience, Heidelberg, Germany), anti-CD11b (BD Bioscience) and anti-Ly49G2 (eBioscience). Subsequently, the slices were stained with secondary antibody and developed with DAB (Vectastain, Vector Laboratories, Burlingame, CA, USA).

Statistics

Where indicated, analysis of significance was performed using the two-tailed Student's t-test (*$p<0.05$; **$p<0.01$). All experiments were performed at least 3 times and representative experiments are shown.

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RESULTS

GDF-15 expression in malignant gliomas *in vivo* translates into elevated serum levels.

To examine the expression of GDF-15 *in vivo*, placenta and gliomas of different WHO grades were stained by immunohistochemistry (Fig. 1A). In normal human white matter tissue specimens (upper right), GDF-15 was detected on endothelial cells of small capillaries (arrow) but not on neuroepithelial cells. Diffuse astrocytomas (WHO grade II, middle left) and anaplastic astrocytomas (WHO grade III, middle right) showed an upregulation of GDF-15 on neoplastic glial cells beside the endothelial expression (arrows). Strongest GDF-15 expression was observed in glioblastomas (WHO grade IV, lower left and right) both on neoplastic astrocytic cells and endothelial cells of vascular proliferations (asterisk). The distribution of GDF-15 was homogeneous within the five samples stained for each tumor entity.

We next aimed at testing whether glioma-derived GDF-15 was also detectable in the blood. To this end, we compared the GDF-15 levels in the sera of glioblastoma patients (n=13) and healthy controls (n=20). In order to avoid confounding effects of therapy-induced elevations of GDF-15, only samples from treatment-naïve patients, that is, without prior radiation- and chemotherapy and without exposure to steroids, were measured by ELISA. This revealed a highly significant elevation of serum GDF-15 levels in the serum of glioblastoma patients ($p<0.01$, unpaired two-sided Student´s t-test) (Fig. 1B).

Human malignant glioma cells express GDF-15 *in vitro.*

We went on to define the expression of GDF-15 in a panel of human glioma cell lines and primary polyclonal glioblastoma cell cultures *in vitro*. Real-time PCR revealed that GDF-15 mRNA expression levels were 10 to 600-fold increased in all of 12 examined glioma cell

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lines and 3 primary glioblastoma cell cultures when compared with normal human brain (Fig. 2A). Immunoblot analysis confirmed the presence of fully processed GDF-15 in 11 of 12 investigated glioma cell supernatants (Fig. 2B). There was a reasonably good correlation of mRNA and protein levels (R^2=0.69) with LN-428 and A172 showing the highest GDF-15 expression on both mRNA and protein level while the low mRNA expression found in U87MG, U251MG and U373MG translated into weak, but still detectable protein levels. The apparent lack of GDF-15 in supernatant from U138MG cells might be due to poor processing, inadequate secretion or to non-specific adhesion of the cytokine to the cell surface (35). In line with previous studies (30), no correlation (R^2=0.05) was found between GDF-15 expression and p53 status of the cell lines (33).

GDF-15 promotes glioma cell proliferation and protects malignant glioma cells from NK and T cell-mediated cytotoxicity *in vitro*

To allow for studies in a syngeneic setting, we confirmed the expression of GDF-15 in supernatant of murine SMA-560 glioma cells. In order to perform loss-of-function experiments, we used RNA interference to stably silence the GDF-15 gene in these cells. Immunoblot revealed a down-regulation of GDF-15 in SMA-560_shGDF-15 pool transfectants by more than 90% as quantified by densitometry (Fig. 3A). Under normal cell culture conditions, this had no apparent effect on growth or morphology of the cells. However, when we investigated the proliferation of these glioma cell sublines under serum-free conditions, SMA-560_shGDF-15 cells incorporated significantly less [methyl-^{3}H]thymidine than the corresponding control-transfected cells. This effect could be reverted by addition of exogenous GDF-15 (5 ng/ml) or FCS (10%) to the GDF-15-depleted cells (Fig. 3B and *data not shown*) whereas addition of GDF-15 to control-transfected cells did not

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further promote their growth. Thus, endogenously produced GDF-15 acts as an autocrine growth factor for glioma cells.

One of the most striking alterations caused by depletion of TGF-β in glioma cells is the increase in tumor cell immunogenicity (10). Therefore, we hypothesized that GDF-15 might also contribute to glioma-derived immunosuppression. Using syngeneic NK cells, we found that SMA-560_shGDF-15 cells were significantly more susceptible to immune cell lysis than control cells (Fig. 3C). In order to investigate the effect of GDF-15 on immune cells other than NK cells, splenocytes were prepared from syngeneic VM/Dk mice and NK cells were depleted by MACS. The cells were cocultured for 5 days with SMA-560_control or shGDF-15 cells and then used as effectors against fresh SMA-560_shGDF-15 targets. SMA-560_shGDF-15 cells were efficiently killed by splenocytes primed with GDF-15-deficient glioma cells whereas priming with SMA-560_control cells induced significantly lower lytic activity (Fig. 3D). Of note, primed lymphocytes do not display cytotoxic activity against syngeneic splenocytes (lymphoblasts) under these conditions. Consistent with an increase of the lytic activity of immune effector cells, splenocytes cocultured with SMA-560_shGDF-15 cells generated higher levels of IL-2 and decreased IL-10 levels compared to splenocytes cocultured with wild-type cells ($p<0.01$) (Fig. 3E). In contrast, there was no difference in IFN-γ levels (data not shown). Control measurements without splenocytes confirmed that supernatant from glioma cells without splenocytes contained only negligible quantities of the respective cytokines: while IL-2 levels were below the detection limit, IL-10 concentrations were 16 pg/ml for control and 28 pg/ml for shGDF-15 cells.

RNA interference-mediated GDF-15 depletion delays the growth of experimental gliomas in syngeneic mice

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Taken together, the effects of GDF-15 on glioma cell proliferation and immune escape strongly suggest that endogenous GDF-15 expression might promote the tumorigenicity of glioma cells. Thus, we assessed the effect of GDF-15 depletion *in vivo*, using the syngeneic SMA-560 VM/Dk mouse model. In line with the *in vitro* data, tumor growth was significantly delayed when GDF-15–depleted rather than control-transfected glioma cells were subcutaneously inoculated in the flank (Fig. 4A; $p<0.05$ from day 10, $p<0.01$ from day 22 until the end of the experiment). Likewise, when SMA-560 cells were implanted stereotactically into the brains of VM/Dk mice, neurological symptoms were first observed in animals carrying control cells. Accordingly, the median survival was prolonged from 19 days with control tumors to 24 days with shGDF-15 tumors ($p<0.01$ by two-sided, unpaired Student´s t-test). Thus, while at day 21 none of the animals with a control tumor was still alive, 83% of the mice with shGDF-15 tumors were still viable (Fig. 4B). Two mice per group were sacrificed during the experiment for histological analyses. Compared to mice with SMA560 control tumours, experimental gliomas derived from shGDF-15 cells displayed an increased infiltration with T cells and macrophages (Fig. 4C). In contrast, numbers of infiltrating NK cells were very low under both conditions (data not shown).

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DISCUSSION

The hallmarks of malignant glioma cells include their ability to deeply penetrate the surrounding healthy tissue and to inhibit anti-tumor immune responses. Together with their resistance to radio- and chemotherapy, these properties translate into a devastating tumor growth and a dismal prognosis. In order to gain a better understanding of the molecular mechanisms leading to the malignant phenotype of these cells, we characterized the role of GDF-15 for proliferation and immune escape of glioma cells. Immunohistochemistry confirmed that the majority of gliomas express GDF-15 *in vivo* (Fig. 1). Compared to gliomas of WHO grades II and III, we noticed an increase of GDF-15 expression and more GDF-15 positive cells in WHO grade IV tumors. These findings apparently contradict the observation from Shnaper et al. (26) who found glioblastoma cells to be GDF-15 negative *in vivo*. However, the polyclonal rabbit serum provided by the Atlas consortium may be the first GDF-15 antibody to give reliable results in immunohistochemistry. Considering that previous studies have also detected GDF-15 mRNA (36) and protein (37) in gliomas, we trust in the validity of our data. These immunohistochemical observations are further supported by GDF-15 expression analyses in established glioma cell lines. As demonstrated in Fig. 2A, GDF-15 transcripts were upregulated in all of 12 permanent glioma cell lines and three primary polyclonal glioblastoma cell cultures compared to normal human brain. Further, GDF-15 protein was also detectable in the supernantant of most investigated glioma cell lines where its expression largely paralleled the mRNA data. Slight alterations might be due to posttranscriptional regulation and differences in secretion of the protein in different cell lines (35). In summary, our results clearly demonstrate that glioma cells express GDF-15. Nevertheless, tumor-associated microglial cells might also make a substantial contribution to the total GDF-15 levels observed in glioma patients, as proposed by others

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(26). A second important finding from our study which had not been observed in a previous publication were the increased serum GDF-15 levels of glioblastoma patients (Fig. 1B). The very clear ($p<0.01$) difference noticed in our study might be due to the fact that we exclusively included treatment-naïve patients thereby avoiding any confounding effects by steroids, chemotherapeutics or irradiation.

In order to define the functional role of glioma-derived GDF-15, we silenced GDF-15 expression in murine SMA-560 glioma cells by RNA interference. This allowed for immunological studies and a functional characterization of GDF-15 in a syngeneic setting (Fig. 3A). We noticed a reduced proliferation of glioma cells that were depleted of GDF-15 (Fig. 3B). However, this effect was not only reverted by addition of recombinant GDF-15 but also by FCS indicating that in complete medium, the lack of GDF-15 is counterbalanced by a multitude of growth factors. In contrast, our immunological studies clearly point out for a novel and until now unrecognized role of GDF-15 as a glioma-derived immunosuppressive molecule. As demonstrated in Fig. 3C, glioma cells depleted of GDF-15 are more susceptible to the lytic activity of NK cells. Likewise, splenocytes from syngeneic mice display improved cytotoxic capacity against SMA-560 targets when primed with shGDF-15 cells rather than with SMA-560 control transfectants. This improved immune cell activation in the absence of GDF-15 is further corroborated by an increase in lymphocyte-derived IL-2 and a decrease in IL-10 in coculture studies (Fig. 3E). These data demonstrate that anti-GDF-15 strategies may partly relieve glioma-induced immunosuppression and enable effective antitumor responses *in vitro*.

In syngeneic mice, subcutaneously injected SMA-560 control cells grew faster than SMA-560 shGDF15 transfectants (Fig 4A). In line with these findings, we observed a survival advantage for mice that had intracranial tumors derived from shGDF-15 cells (Fig. 4B). These findings are in contrast to an earlier publication which described that GDF-15-

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transfected LNZ-308 glioma cells lost tumorigenicity in nude mice (30). However, this model is not only unsuitable for the detection of immunological tumor-host interactions. Ectopic overexpression of GDF-15 may further lead to unphysiologically high cytokine levels that could affect angiogenesis (38). Thus, down-modulation of endogenously expressed GDF-15 in an immune-competent orthotopic mouse model (as described here) is likely to reflect the actual role of GDF-15 in glioma more accurately. Further evidence for the presumed immunomodulatory role of GDF-15 comes from the observation that GDF-15 depleted tumors show stronger infiltration with T cells and macrophages (Fig. 4C). This might, however, be a double-edged sword, since infiltration with regulatory T cells (39) or the presence of tumor-associated macrophages (40) have been linked to a poor outcome in other tumor entities. In brain tumors, however, the presence of T_{reg} appears to be prognostically neutral within the entity of glioblastomas (41). Likewise, no correlation was found between the total number of tumor-infiltrating macrophages and prognosis (42) whereas a (rarely observed) severe lymphocytic infiltration was linked to a significantly longer survival (43). The magnitude of T-cell infiltration may be inversely proportional to intratumoral TGF-β2 levels and correlates positively with clinical outcome (44) – which bears some resemblance to our *in vivo* data on the divergent TGF-β superfamily member GDF-15. The fact that even mice with GDF-15-depleted tumors died suggests that the lack of GDF-15 might have been compensated by other immune-inhibitory mechanisms such as TGF-β. In addition, a selection process for glioma cell clones with a less substantial knock-down of GDF-15 expression might have occurred. Unfortunately, due to a lack of adequate reagents this hypothesis cannot currently be tested by immunhistochemical analysis of mouse tumors. Finally, GDF-15 may also have been provided by host cells, e.g. by microglia in the tumor microenvironment. However, the current lack of knowledge regarding the GDF-15 receptor and its associated signaling mediators do not only limit the mechanistic

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understanding of the involved processes. They also preclude experimental pharmacological approaches like the inhibition of signaling at the receptor level or the specific blockade of GDF-15 dependent signaling cascades. Nevertheless, the reasonably mild phenotype displayed by GDF-15 knock-out mice (45) suggests that such strategies might be more safely directed towards GDF-15 than towards TGF-β, where the targeting of receptor kinases (46) has given rise to serious concerns regarding the safety of this approach. Overall, our study clearly demonstrates that GDF-15 confers immune privilege to malignant gliomas, contributes to the malignant phenotype of these cells and thus represents a novel and promising target for future therapies.

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Figure legends

Fig. 1. **Human malignant gliomas express GDF-15 *in vivo.***

A. Paraffin-embedded tissue sections of normal human white matter (upper right), diffuse astrocytomas (WHO grade II, middle left), anaplastic astrocytomas (WHO grade III, middle right) and glioblastomas (WHO grade IV, lower left and right) were assessed by immunohistochemistry. Normal human placenta (upper left) served as positive control. Syncytiotrophoblasts and endothelial cells which are both expected to express GDF-15 are indicated by arrowheads and arrows, respectively. Five specimens from each entity were stained. Representative stainings are shown (original magnification 200x for all photomicrographs). B. GDF-15 levels in sera of treatment-naïve glioblastoma patients (n=13) and healthy controls (n=20) were determined by ELISA. Median values are indicated. (** denotes $p<0.01$ as assessed by two-sided, unpaired Student´s t-test).

Fig. 2. **Malignant glioma cells express GDF-15 *in vitro.***

A. GDF-15 expression was analysed by real-time RT-PCR using 18S RNA as a reference, with quantitative data expressed relative to normal brain cDNA. B. GDF-15 levels in cell culture supernatants (20 µg/lane) were assessed by immunoblot.

Fig. 3. **GDF-15-depleted glioma cells are more susceptible to immune cell-mediated cytotoxicity *in vitro*.** A. Cell culture supernatants of SMA-560 control or shGDF-15 cells (20 µg/lane) were assessed for GDF-15 protein levels by immunoblot. B. The growth rates of SMA-560 control or shGDF-15 cells were examined by [methyl-^{3}H]thymidine incorporation for 16 h. C. Lytic activity of mouse NK cells against syngenic SMA-560 control (filled diamonds) or shGDF-15 (open squares) cells was assessed in a 4 h ^{51}Cr release assay. D.

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DX5-depleted syngeneic splenocytes were co-cultured (primed) with SMA-560 control or shGDF15 cells for 5 days. The lytic activity of primed splenocytes against SMA-560 shGDF-15 cells was determined in a 4 h ^{51}Cr release assay. E. Cytokine levels in cocultures of DX5-depleted splenocytes with SMA-560 control or shGDF-15 glioma cells were determined by ELISA.

Fig. 4. **RNA interference targeting GDF-15 delays the growth of subcutaneous and intracerebral gliomas in syngeneic immunocompetent mice.** A. VM/Dk mice were subcutaneously injected with 10^5 SMA-560 control (filled squares) or shGDF-15 (open triangles) cells. Tumor size was measured using a caliper every 3 days. B. SMA-560 control or siGDF15 transfectants were inoculated intracerebrally in syngenic VM/Dk mice. Survival data for 6 animals per group are presented as Kaplan-Meier plot. C. At day 15 after tumor inoculation, 2 animals were sacrificed. Their brains were removed, shock-frozen and assessed for the infiltration of T cells and macrophages. Representative stainings are shown.

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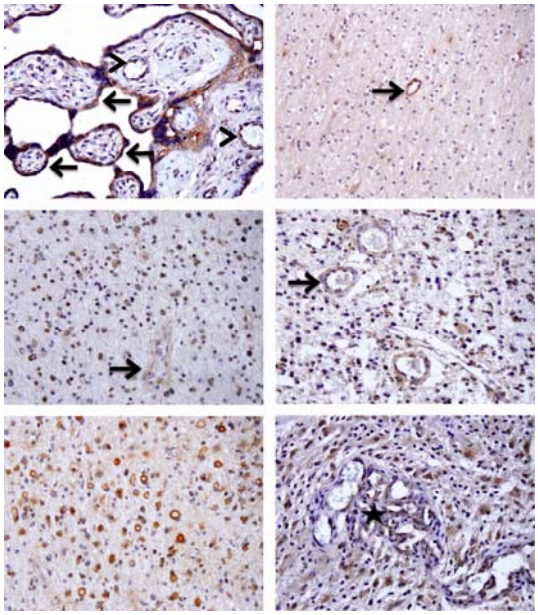
Fig. 1

A



B

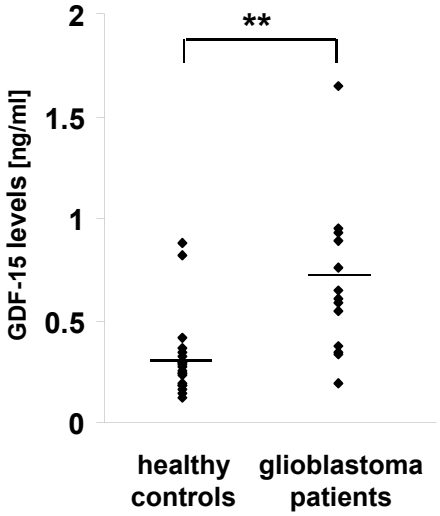


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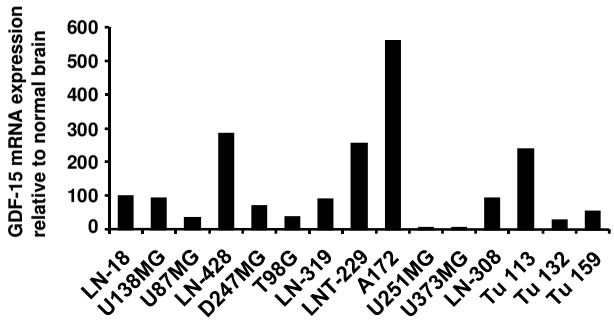
Fig. 2

A



B

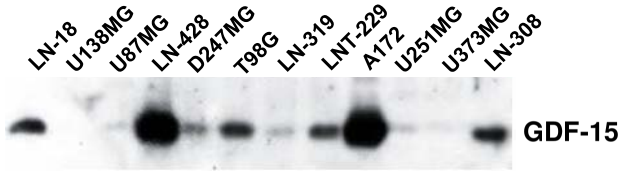


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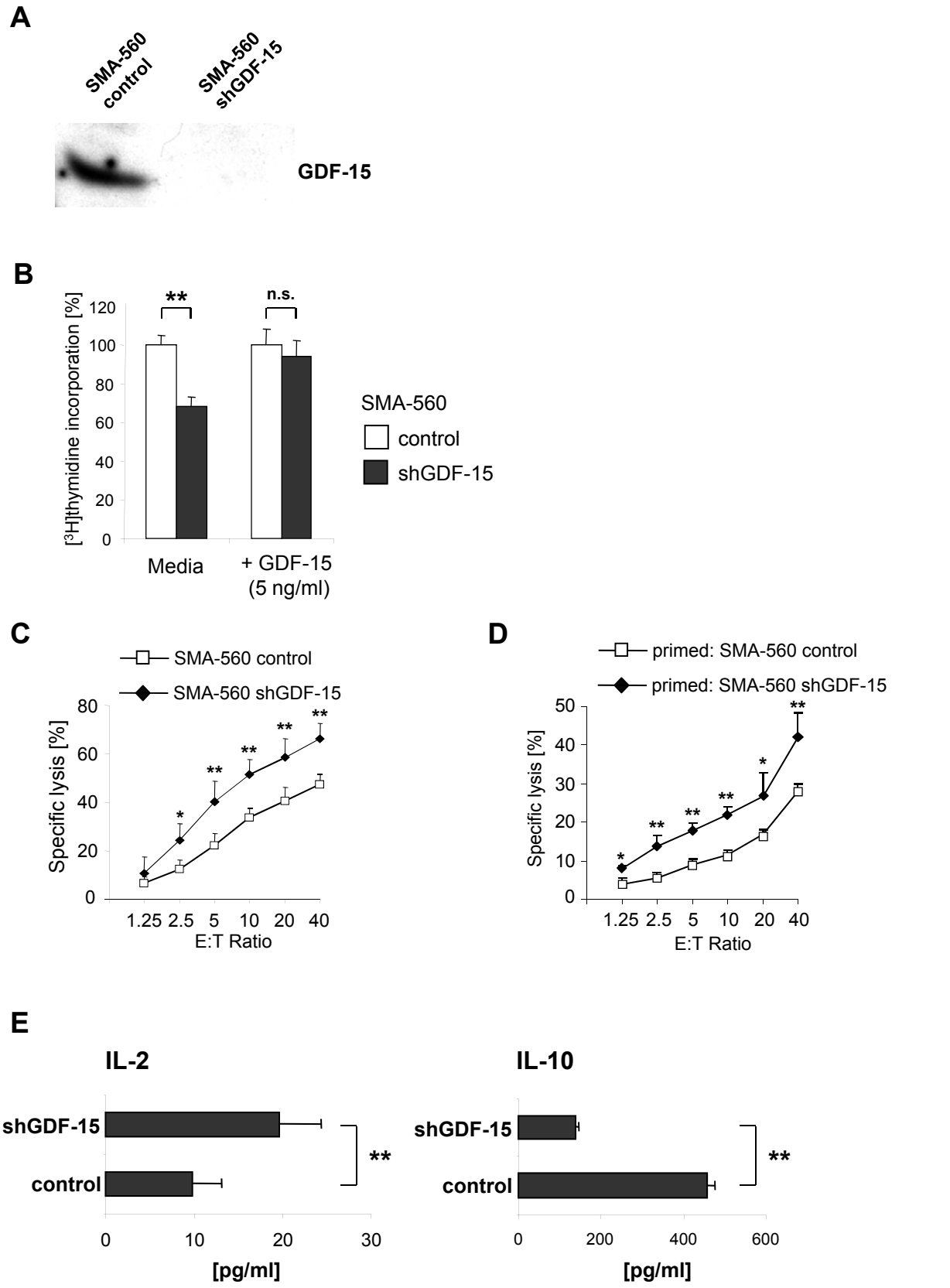
Fig. 3



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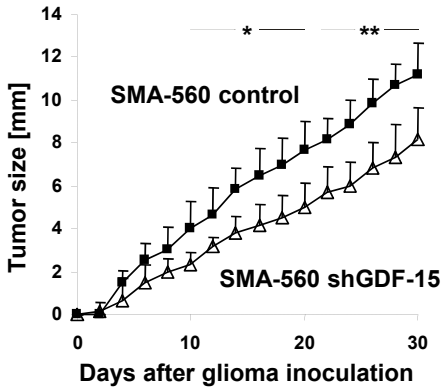
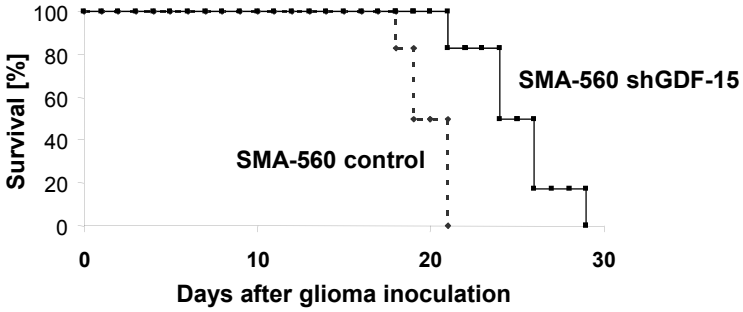
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig. 4

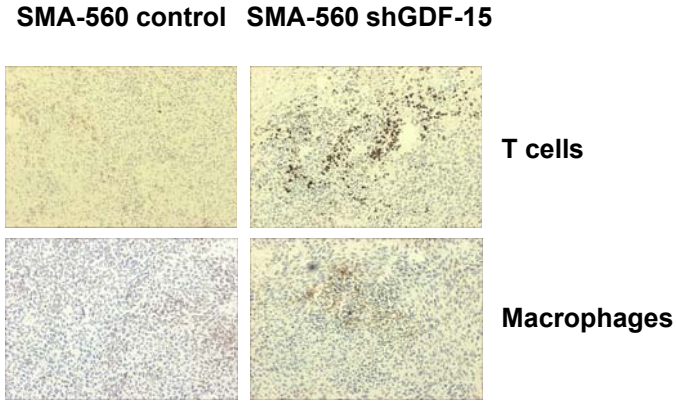
A



B



C



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